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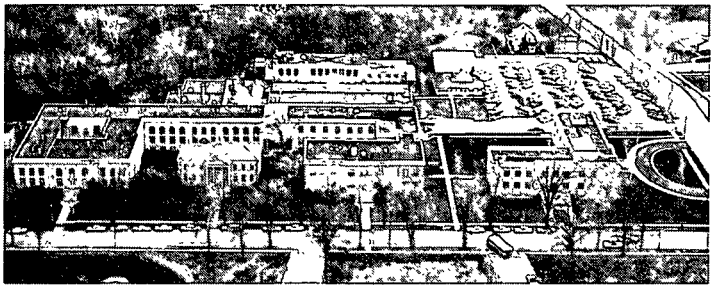

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NORWAY SPRUCE SOMATIC EMBRYOGENESIS: HIGH FREQUENCY INITIATION FROM LIGHT CULTURED MATURE EMBRYOS

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Norway Spruce Somatic Embryogenesis: High Frequency Initiation from Light Cultured Mature Embryos

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Norway spruce somatic embryogenesis: high frequency initiation from light cultured mature embryos

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Key words: somatic embryogenesis, regeneration, tissue culture, Picea abies, glutamine, organic nitrogen.

Abstract. Somatic embryos and rooted plantlets have been regenerated from light-initiated embryogenic callus derived from mature embryos of Picea abies stored seeds. Under a 16 hour photoperiod, mature zygotic embryos were cultured on a modified half-strength Murashige-Skoog medium without NH_4NO_3 and supplemented with 5 mM glutamine, 4.5 µM N6-benzyladenine (BA) and 10.7 µM naphthalene-acetic acid (NAA) or 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D). White translucent embryogenic callus, proliferating from the callusing hypocotyl region after 3 weeks incubation, was isolated from the green non-embryogenic tissue and subcultured for over 12 months. Upon transfer of the embryogenic callus to medium containing abscisic acid (ABA) and indole-3-butyric acid (IBA), somatic embryos proceeded to mature, elongating and forming a ring of cotyledonary leaves similar to those of a zygotic embryo. Transferred to medium without growth regulators, the somatic embryos "germinated", producing plantlets with green cotyledons, elongated hypocotyls and primary roots.

Introduction

Conifer cell and tissue culture has advanced rapidly in recent years. Regeneration had previously been limited to organogenic shoot induction from very juvenile explants. In 1985 plantlet regeneration via somatic embryogenesis was reported in both Norway spruce [1,2] and European larch [3]. Successful initiation of embryogenic callus required explants at specific developmental stages from immature seeds. This "time" element placed severe constraints on continuing initiation studies of conifer embryogenesis. Reports of somatic embryogenesis from mature seed embryos of Norway spruce [4-6] and sugar pine [7] followed these first accomplishments. Initiation of embryogenic callus from mature embryo explants of Norway spruce, however, has been reported to occur only under culture in the dark [4,5] or at very low frequencies in the light [8]. We present an alternate and reproducible method of generating embryogenic callus at high frequency under a 16 hour photoperiod from excised mature zygotic embryos of Norway spruce.

Materials and methods

Explants

Norway spruce seeds were obtained from Quality Tree Seed, Inc., Brewster, N.Y. in 1985 and stored at 4°C. Prior to excision of the embryos, seeds were surface sterilized in 30% H_2O_2 for 45 minutes and rinsed 3 times in sterile water. After imbibing overnight in sterile water, embryos were asceptically dissected from the gametophytic tissue and cultured on solidified media in 15 x 100 mm culture dishes, 5 embryos/plate. Thirty to fifty embryos were cultured for each treatment in all trials. Initiation frequency was determined by scoring the number of embryos producing embryogenic callus.

Media and culture

Two basal media variations, BLG and HM, were used in all studies (Table 1). Embryogenic callus was initiated and maintained on 0.5 BLG basal medium (9) supplemented with either 10.7 μM NAA or 10 μM 2,4-D and 2.2-22.5 μM BA. This half-strength modified MS medium omits NH_4NO_3, decreases KNO_3 to 0.5 mM and replaces the ammonium with 5 mM of L-glutamine. The medium was adjusted to pH 5.7, gelled with 0.7% Bacto agar and autoclaved before adding the filter-sterilized glutamine to the cooled medium. Initiation was attempted on both full and half strength BLG as well as on "HM", a modification of basal medium used by Hakman et al. to induce somatic embryogenesis from immature zygotic embryos [1]. All cultures were initiated and maintained at 23 ± 1°C with 16 hr irradiance (10 to 40 μE m^{-2} s^{-1} at culture level) from cool-white fluorescent lights. Embryogenic callus was subcultured at 21 day intervals.

Embryo development

Somatic embryo development was achieved by the method of Becwar et al. [10,11]. Briefly, callus was transferred to HM medium without growth regulators for 1 week, followed by transfer to HM containing 1 μM each ABA and IBA. Half-strength BLG was also tested as a basal medium in this serial transfer. Basal medium without growth regulators promoted embryo elongation and root development prior to transfer of plantlets to soil.

Results and discussion

Embryogenic callus initiation

After two weeks in culture, 40-50% of the explants began to callus just below the green swelling cotyledons. At 3 weeks, isolated somatic embryos were often observed developing from the callusing hypocotyl area (Fig 1a). These low-frequency embryos continued to proliferate producing fast growing embryogenic

masses (Fig 1b). Most of the embryogenic callus, however, was first observed as areas of white, glassy tissue on or around the callusing explant (Fig 1c,d). By 4-6 weeks, the phenotypically embryogenic tissue had formed a gelatinous and filamentous mass of clear polarized structures. After isolation from the surrounding green or tanning callus, this tissue has been maintained through serial subculture for over 1 year (Fig 1e).

Media effects on initiation

The influence of basal media and growth regulators on initiation of embryogenic callus is shown in Table 2. The greatest number of embryogenic lines capable of long term culture in all trials was consistently generated on 0.5 BLG containing 10.7 μM NAA or 10 μM 2,4-D plus 4.5 μM BA. The higher BA level, 22.5 μM, stimulated earlier appearance of embryogenic-type callus; however, this concentration limited the number of lines that could be maintained over time. The response to full and half-strength basal media components was also compared. In one trial of 50 replicates, 28% of the embryos cultured on 0.5 BLG produced embryogenic callus that was maintained for over 6 months. From the same test, 50 explants on full-strength BLG medium supplemented with the same growth regulators produced only subculturable green compact, non-embryogenic callus. HM medium which induces embryogenesis from immature embryos under light conditions, produced embryogenic callus from mature embryos at a very low frequency, and could not be maintained. Half-strength HM medium failed to produce any embryogenic tissue by 8 weeks compared to 21% (10 lines) initiated on 0.5 BLG when run concurrently with the same growth regulators under the same photoperiod.

High frequency embryogenic callus initiation from mature embryos under a 16 hr photoperiod contrasts to the dark culture requirement reported by other

investigators. In their early work, von Arnold and Hakman obtained embryogenic callus from mature Norway spruce embryos on full strength medium with a 1% sucrose level when cultured in the dark [4]. More recently, improved efficiency was reported by culturing embryos in the dark on half-strength medium but with full levels (15 mM) of NH_4NO_3 and 1% sucrose [8]. When cultured in the light, 50% of the embryo explants were reported to form adventitious buds, and less than 0.5% produced embryogenic callus. Gupta and Durzan [5] also reported that darkness and 2,4-D were essential for induction of somatic embryogenesis from mature Norway spruce embryos. In our experiments, NAA and 2,4-D in half-strength basal medium with no NH_4NO_3 and 1% sucrose initiated embryogenic callus equally well under a light photoperiod.

In this report, successful initiation of embryogenic callus under a 16 hr photoperiod may be due to the replacement of inorganic NH_4NO_3 with an organic form of reduced nitrogen (glutamine) in the basal medium. In related work, we replaced glutamine, asparagine and KCl levels in 0.5 BLG with 0.5 levels of NH_4NO_3 and KNO_3 as supplied in the HM formulation. Mature Norway spruce embryos were excised to control and test media, and half of each treatment was incubated in total darkness and half in a 16/8 light cycle. At 5 weeks, the percent of embryogenic lines initiated on each medium was not significantly different from one another regardless of photoperiod (Table 3). The numbers of embryogenic lines initiated under both photoperiods on 0.5 BLG medium containing the organic nitrogen were, however, more than 3 times the total number generated on the inorganic nitrogen medium. Furthermore, only lines initiated with the organic nitrogen (glutamine) could be maintained under both light and dark conditions.

Somatic embryo development

Continued development of somatic embryos is cell line dependent. Four callus lines, each derived from different embryo explants, were transferred to the developmental sequence [10,11] using both HM and 0.5 BLG as basal media. After 3-4 weeks on the HM medium, multiple smooth and dense embryonal heads were clearly visible in the callus masses of 1 of the 4 cell lines (Fig 2a). Within 30-45 days from transfer to HM + charcoal, 2-4 of the approximately 50 potential embryos per callus mass (about 100 mg fresh weight) elongated and developed cotyledons (Fig 2b,c). In another trial, well developed somatic embryos were generated from 8 of 10 calli from this line. No development occurred from any tissue transferred via the same sequence with 0.5 BLG as the basal medium. Transfer of the maturing somatic embryos to half-strength MS medium without growth regulators promoted development to complete plantlets with green cotyledons, elongating hypocotyls and primary root growth (Fig 2d).

Summary

To our knowledge, this is the first report of high frequency initiation of embryogenic callus from mature Norway spruce embryos cultured under a 16 hr photoperiod. Our results point to an interaction between light and reduced nitrogen components of the initiation medium. Half-strength BLG medium components were required to avoid exclusive proliferation of green, nonembryogenic callus. The newly emerging embryogenic tissue, in contrast to a recent report [5], appears to arise from the callusing hypocotyl area in a manner similar to that observed from immature zygotic embryos [12]. Plantlets have been regenerated from the somatic embryos, and efforts are continuing to increase the frequency of conversion.

Acknowledgement

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Table 1. Basal media formulations, mg/l

Components	BLG	HM
NH_4NO_3	--	1200
KNO_3	100	1900
$MgSO_4 \cdot 7H_2O$	320	370
KH_2PO_4	170	340
$CaCl_2 \cdot 2H_2O$	440	180
KCl	745	--
KI	0.83	0.75
H_3BO_3	6.2	0.63
$MnSO_4 \cdot H_2O$	16.9	2.2
$ZnSO_4 \cdot 7H_2O$	8.6	2.87
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.025
$CuSO_4 \cdot 5H_2O$	0.025	0.0025
$CoCl_2 \cdot 6H_2O$	0.025	0.0025
$FeSO_4 \cdot 7H_2O$	27.8	13.9
Na_2EDTA	37.3	18.65
Inositol	100	100
Nicotinic Acid	0.5	2
Pyridoxine	0.1	1
Thiamine HCl	0.1	5
Sucrose	20000	34200
Glucose	--	180
Xylose	--	150
Arabinose	--	150
Glutamine	1500	--
Asparagine	100	--
Agar	0.7%	0.5%
pH	5.8	5.5

Table 2. Effect of media and growth regulators on initiation and maintenance of embryogenic callus from mature Norway spruce embryos

Basal Media	Auxin/Cytokinen µM			Embryogenic Lines % Explants[a]		
	NAA	2,4-D	BA	4-6 wks	8-10 wks	4-6 mo
0.5 BLG	10.7		4.5	28	18	16
0.5 BLG	10.7		22.5	32	14	7
BLG	10.7		4.5	0	3	0
0.5 BLG		10	4.5	21	16	11
HM		10	4.5	1.5	0	0
0.5 HM	10.7		4.5	0	0	0

[a]Data were pooled from 3 separate experiments of 30-50 explants per treatment.

Table 3. Effect of photoperiod and nitrogen components on initiation of Norway spruce embryogenic callus at 5 weeks

Media	% Embryogenic Callus, N = 50	
	Light, 16 hr	Dark, 24 hr
0.5 BLG Control	20^{AB}	30^{A}
0.5 BLN[a]	4^{B}	10^{B}

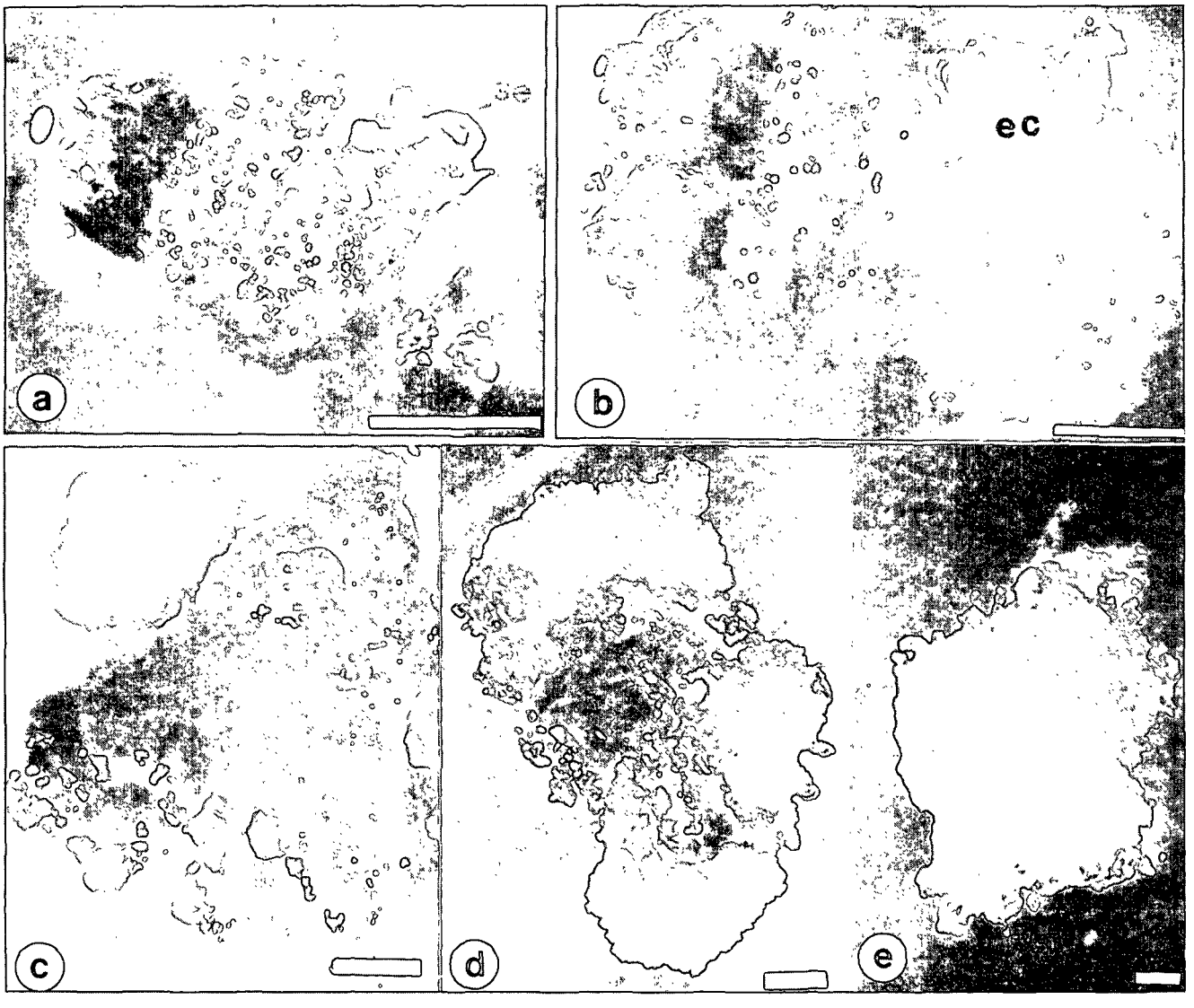
[a]Glutamine asparagine and KCl of control medium were replaced with 600 mg/l NH_4NO_3 and 950 mg/l KNO_3.

Values with a common superscript are not significantly different from one another ($P < 0.05$); number of replications is indicated by N.

Figure Captions

Fig. 1. Embryogenic callus initiated from mature Norway spruce embryos cultured under 16 hour photoperiod on 0.5 BLG medium supplemented with 10.7 μM NAA and 4.5 μM BA. (a) Somatic embryos (se) arising from callusing hypocotyl area. (b) Same explant after 10 days. Embryogenic callus (ec) is proliferating from the low-frequency somatic embryos shown in (a). (c,d) Embryogenic callus proliferation following 4-6 weeks in culture. (e) Embryogenic callus subcultured over 1 year. Bar = 0.1 mm.

Fig. 2. Plantlet development from light-initiated and subcultured embryogenic callus derived from mature zygotic Norway spruce embryos. (a) Smooth embryonal "heads" and early somatic embryos (se) emerging from callus 21-28 days after transfer to HM medium with 1 each ABA and IBA. (b,c) Maturation of somatic embryos (elongating hypocotyls and development of cotyledonary nodes). (d) Plantlet development following transfer of matured embryo to basal medium without growth regulators. Bar = 1 mm.



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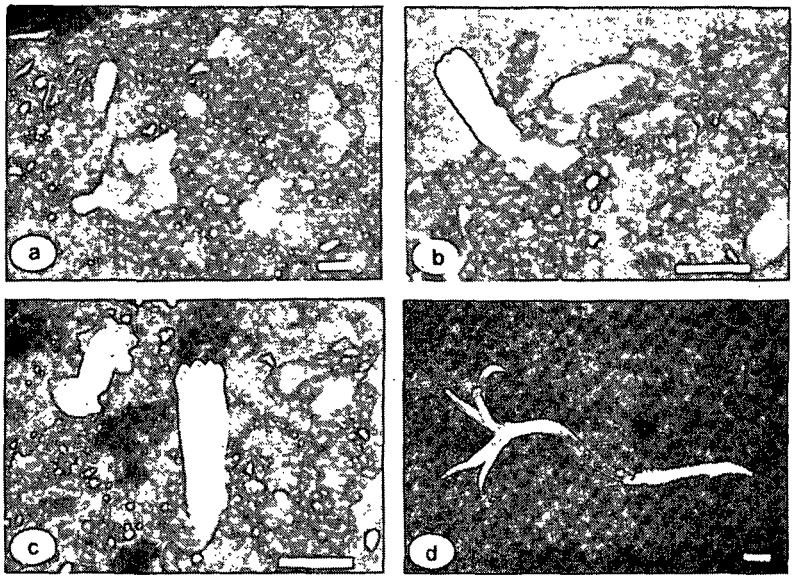

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